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Acquisition of an *Agrobacterium* Ri Plasmid and Pathogenicity by Other α-*Proteobacteria* in Cucumber and Tomato Crops Affected by Root Mat

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Root mat of cucumbers and tomatoes has previously been shown to be caused by *Agrobacterium radiobacter* strains harboring a root-inducing Ri plasmid (pRi). Nine other pRi-harboring α-*Proteobacteria* have subsequently been isolated from root mat-infected crops. Fatty acid profiling and partial 16S rRNA sequence analysis identified three of these strains as being in the genus *Ochrobactrum*, five as being in the genus *Rhizobium*, and one as being in the genus *Sinorhizobium*. An in vitro pathogenicity test involving inoculation of cucumber cotyledons was developed. All pRi-harboring α-*Proteobacteria* induced typical root mat symptoms from the cotyledons. Average transformation rates for rhizogenic *Ochrobactrum* (46%) and *Rhizobium* (44%) strains were lower than those observed for rhizogenic *A. radiobacter* strains (64%). However, individual strains from these three genera all had transformation rates comparable to those observed from cotyledons inoculated with a rhizogenic *Sinorhizobium* strain (75%).

Since the early 1990s, hydroponic cucumber and tomato crops in the United Kingdom and some other European countries have been affected by a disorder known as root mat. In affected crops symptoms are expressed as extensive root proliferation within rockwool cubes and slabs with, in severe cases, losses in marketable yield. An earlier outbreak of root mat in the 1970s, in soil and straw bed cucumber crops, had been associated with the presence of *Agrobacterium* biovar 1 strains (*Agrobacterium radiobacter*). In the late 1990s, surveys of affected crops and subsequent host tests with *Agrobacterium* strains isolated during the surveys showed that the disease in cucumbers, and a similar disorder in tomatoes, was caused by *A. radiobacter* strains harboring a root-inducing Ri plasmid (pRi) (32, 33).

Root mat symptoms are induced following transfer and expression of a segment of pRi DNA (T-DNA) into the plant cell genome. Unlike some other tumor-inducing Ti plasmids (pTi) and pRi, which have two distinct T-DNA fragments, the T-DNA of a root mat-associated (RMA) pRi comprises a single continuous T-DNA fragment (24). The T-DNA of pRi and pTi in transformed plant tissues encode genes that induce cell proliferation and also synthesis genes for unusual amino acid derivatives or sugar-phosphodiesters. These compounds are termed opines and are catabolized by genes also present on the infecting pRi or pTi (12). The opine associated with RMA pRi has been termed cucumopine (5).

The taxonomy of *Agrobacterium* is confused. The majority of *Agrobacterium* species form a phylogenetic clade that may include some related organisms, such as *Rhizobium galegae*, while a number of pRi- or pTi-containing strains, known as *Agrobacterium rhizogenes* or biovar 2, form a group that is phylogenetically within the genus *Rhizobium* (see Fig. 2). Although a proposal has recently been made to incorporate all *Agrobacterium* species into the genus *Rhizobium* (37), we have retained the established nomenclature because the majority of *Agrobacterium* species, including the recognized root mat pathogen *A. radiobacter* biovar 1, are phylogenetically distinct from *Rhizobium* spp.

During the surveys of the late 1990s, bacterial strains had been isolated from cucumber and tomato root samples plated onto either nutrient dextrose medium or an *Agrobacterium* biovar 1 semiselective medium (23) and had been identified by fatty acid profiling (26), which is known to differentiate many taxa within *Agrobacterium* and other genera of the α-*Proteobacteria* (28). Strains with fatty acid profiles similar to those obtained from known *A. radiobacter* were tested by the PCR protocols of Haas et al. (11), which provide a tentative discrimination between pTi and pRi. Strains with fatty acid profiles not consistent with known *A. radiobacter* strains were stored (at −80°C) but not tested for the presence of the plasmid, as it was assumed that rhizogenic *A. radiobacter* strains were the causal agent of the disorder.

Experimental transfer of *Agrobacterium* pTi via conjugation to recipient α-*Proteobacteria* in other genera has been reported previously (13, 27, 31). Hooykaas et al. (13) reported that *Rhizobium leguminosarum* biovar *trifolii* transconjugants were able to induce tumors on inoculated host plants.

This paper reports the isolation, from RMA cucumber and tomato crops, of pRi-harboring α-*Proteobacteria* which do not belong to the genus *Agrobacterium*. An in vitro hairy root culture assay was modified to act as a rapid host test to determine if these strains possessed the ability to induce typical root mat symptoms on inoculated cucumber cotyledons.

MATERIALS AND METHODS

Detection of pRi DNA in putative non-*Agrobacterium* strains. Over 300 putative non-*Agrobacterium* strains, isolated from affected cucumber and tomato crops since 1997, were removed from a cryogenic (−80°C) storage system (Pro-

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TABLE 1. Characterization and pathogenicity of pRi-harboring α-*Proteobacteria* and representative *Agrobacterium* strains isolated from RMA cucumber and tomato crops

Strain(s)	Identification[a]	Source	PCR[b] result for:			Cucumber hairy root culture[c]
			fliG	*virD2*	*rol*	
CSL 2411	*Rhizobium* sp.	Cucumber	−	+	+	Positive (5/11, 45.5%)
CSL 2412	*Rhizobium* sp.	Cucumber	−	+	+	Positive (2/14, 14.3%)
CSL 2542	*Rhizobium* sp.	Cucumber	−	+	+	Positive (4/11, 36.4%)
CSL 2573	*Ochrobactrum* sp.	Cucumber	−	+	+	Positive (8/12, 66.6%)
CSL 2611	*Sinorhizobium* sp.	Cucumber	−	+	+	Positive (9/12, 75%)
CSL 2637	*Ochrobactrum* sp.	Cucumber	−	+	+	Positive (3/12, 25%)
CSL 3809	*Ochrobactrum* sp.	Tomato	−	+	+	Positive (5/11, 45.5%)
CSL 4520	*Rhizobium* sp.	Cucumber	−	+	+	Positive (9/13, 69.2%)
CSL 4733	*Rhizobium* sp.	Cucumber	−	+	+	Positive (7/13, 53.8%)
CSL 3260	*A. radiobacter*	Cucumber	+	−	−	Negative (0/9)
All rhizogenic *Agrobacterium* strains	*A. radiobacter*	Cucumber	+	+	+	Positive (47/74, 63.5%)

[a] Identification based on fatty acid profiling and 16S rRNA sequence analysis.
[b] For more information on procedures for *fliG* PCR, see Weller et al. (36); for *virD2* PCR, see Haas et al. (11); for *rol* PCR, see Weller and Stead (35).
[c] Positive results are from the total number of discs surviving in culture for at least 14 days.

tect Bacterial Preservers; Technical Service Consultants, Heywood, Lancashire, United Kingdom) and grown overnight on nutrient agar at 28°C. DNA was extracted from resulting cultures by heating a 100-μl suspension of each culture (6 min, >95°C). A supernatant was obtained from this suspension by centrifugation (14,500 rpm, 3 min) (Eppendorf MiniSpin plus; Eppendorf AG, Hamburg, Germany) and used as template in two PCR assays, the conventional PCR of Haas et al. (11), testing for the *virD2* region found in all pTi and pRi, and the *rol* TaqMan PCR (35), testing for a gene commonly found in the T-DNA of RMA pRi.

Strains positive for both PCR assays were tested by the *fliG* TaqMan assay (36), an assay specific to *Agrobacterium* spp., using the original DNA extract as template. Strains were then reidentified by fatty acid profiling (26). Strains were cultured at 28°C for 24 ± 1 h on Trypticase soy agar, and saponification, methylation, and purification of fatty acid methyl esters was carried out in a single test tube. Fatty acid methyl esters were separated by the MIDI microbial identification system (Newark, N.J.) utilizing a Hewlett Packard HP 6890 series gas chromatograph. Profiles were compared with those stored in the MIDI TSBA 40 and in-house National Collection of Plant Pathogenic Bacteria (NCPPB; Central Science Laboratory [CSL], Sand Hutton, York, United Kingdom) libraries. The former comprises a wide range of species of aerobic bacteria, the latter comprises taxa of plant-pathogenic bacteria and is based on strains housed in the NCPPB.

Strain characterization by 16S rRNA sequence analysis. A DNA extract was prepared from rhizogenic strains which did not have fatty acid profiles consistent with known *Agrobacterium* species and had tested negative in the *fliG* PCR assay with a genomic DNA extraction kit (Wizard genomic DNA purification kit; Promega Corporation, Madison, Wis.). These extracts were used for partial 16S rRNA sequencing. Sequencing was done directly from a 1,050-bp PCR product amplified by the primers fA (5′-GGAGAGTTAGATCTTGGCTCAG) and rG (5′-CCCCACCTTCCTCTCGGCTTATC) (2). The PCR product was purified (Wizard SV Gel and PCR Clean-Up System; Promega Corporation) and sent to the University of York Technology Facility for sequencing. The PCR product was sequenced from the forward (fA) and reverse (rG) primers to produce a consensus sequence for the 1,050-bp product. The resulting sequence was compared to similar sequences in the GenBank database via a BLAST search.

In vitro pathogenicity test. The cucumber hairy root culture protocol of Shi et al. (25) was adapted as follows. Cucumber seeds (cv. Jessica) were surface disinfected overnight in a 5% plant preservative mixture solution (Plant Cell Technology Inc., Washington, D.C.). Disinfected seeds were germinated on hormone-free Murashige and Skoog basal medium (MS) (17) containing 3 g of Phytagel (Sigma-Aldrich Ltd., Poole, Dorset, United Kingdom) per liter at 25°C, 16 h of light. Seedlings were harvested 6 to 10 days postgermination. Cotyledons were removed and cut at the basal end of the leaf (approximately 1-cm cut width) and cultured overnight on hormone-free MS medium at 25°C. Disks were then inoculated with a liquid MS medium containing the bacterial strain to be tested (5-μl loop of overnight culture in 10 ml) by floating the disks on this suspension in a petri dish. After 45 min the disks were placed back on the original MS medium for 48 to 72 h at the original culture conditions. Disks were then transferred onto MS (plus 100 mg of cefotaxime/liter) medium, and root formation was induced at 28°C, 16 h of light. Disks were inoculated with a panel of rhizogenic *A. radiobacter* strains and all other α-*Proteobacteria* strains shown to harbor pRi DNA. In all protocols disks were inoculated with liquid MS medium and a rhizogenic *A. radiobacter* strain (NCPPB 4042) to serve as negative and positive controls, respectively. Plates were observed for the presence of roots every 3 to 4 days.

Extraction of root DNA and analysis by PCR. To demonstrate the validity of the root culture assay and to indicate that roots, induced from inoculated disks and showing root mat morphology, did contain transformed tissue, DNA was extracted from 0.2 g of root material induced from a cucumber cotyledon inoculated with the rhizogenic *A. radiobacter* NCPPB 4042 strain (5 weeks after inoculation) by using a DNA extraction kit (Wizard magnetic DNA purification system for food; Promega Corporation). This extract was used as template in the *Agrobacterium fliG* and the pRi T-DNA *rol* PCR assays as well as the internal control COX TaqMan PCR assay (34), designed within the published sequence (22) of the constitutive cytochrome oxidase (COX) plant gene.

Nucleotide sequence accession numbers. The 16S rRNA sequence data cited in this study were deposited in GenBank under accession numbers AY306216 to AY306221 and AY306224 to AY306228.

RESULTS

Identification of rhizogenic non-*Agrobacterium* spp. Nine strains were isolated that tested positive in each of the pRi PCR assays, tested negative in the *fliG* assay, and had fatty acid profiles that indicated they were not in the genus *Agrobacterium* (Table 1). Phylogenetic analysis of fatty acid data indicated that although these strains do not belong to a known *Agrobacterium* species, they were closely related to the genus *Agrobacterium* and were thus termed α-*Proteobacteria* strains (Fig. 1).

Strain characterization by partial 16S rRNA sequencing. Partial 16S rRNA sequence analysis of the nine rhizogenic α-*Proteobacteria* strains indicated that, according to a BLAST search on the GenBank database, these strains were not in the genus *Agrobacterium*. 16S rRNA sequences of representative α-*Proteobacteria* were obtained and edited so that the first 11,00 bp of sequence from the position of the fA primer could be aligned with the 16S sequences of the rhizogenic strains, including two pRi-harboring *A. radiobacter* strains (CSL 3276 and NCPPB 4042) which had been similarly edited. Phylogenetic analysis of these sequences showed that none of the nine rhizogenic α-*Proteobacteria* strains belonged to the genus *Agrobacterium*, with strains falling into two main clusters. One cluster of five strains (CSL 2411, 2412, 2542, 4520, and 4733) formed a clade with two *Rhizobium* spp., and one cluster of

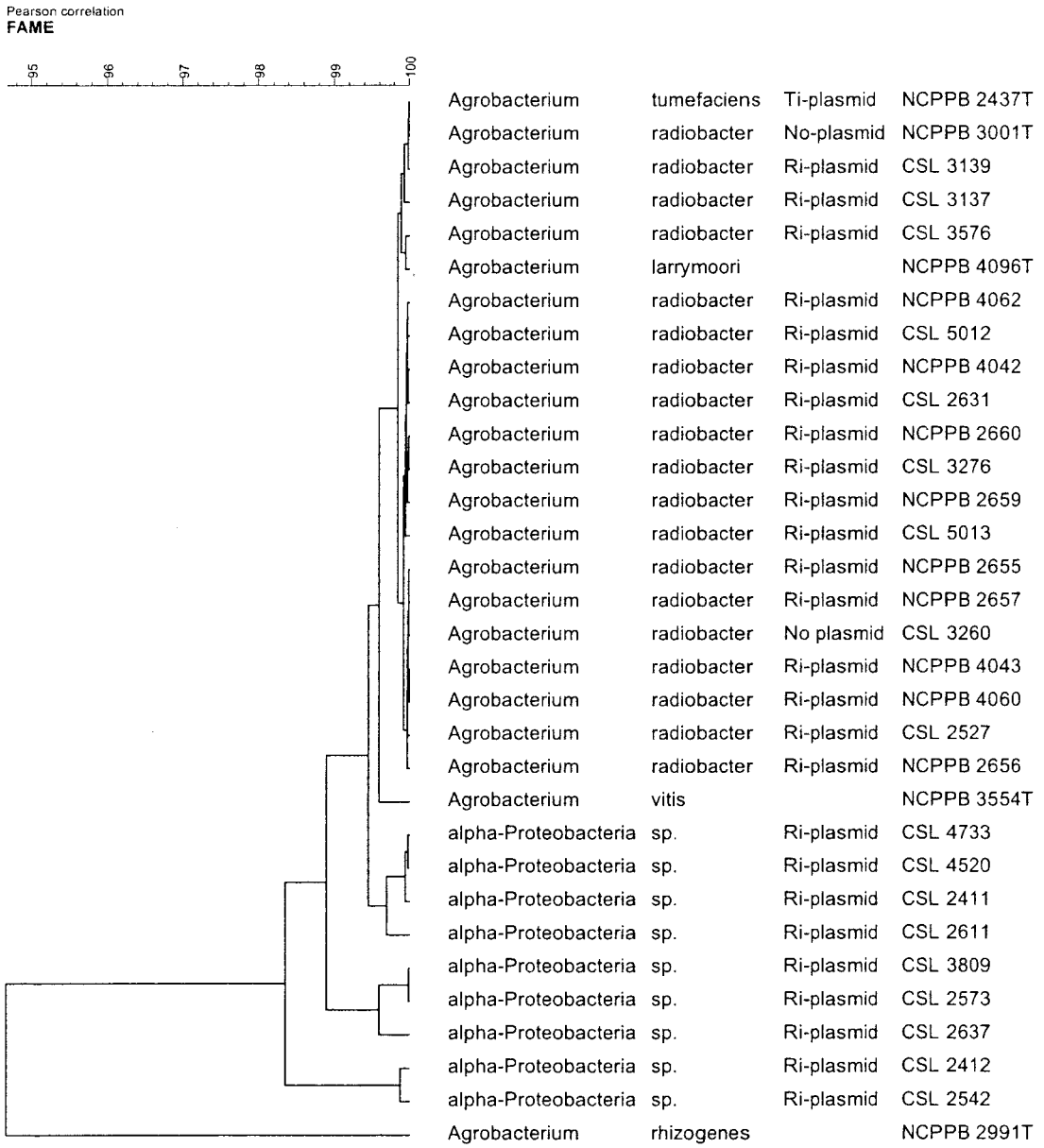

FIG. 1. UPGMA dendrogram for fatty acid methyl ester data from a panel of RMA *A. radiobacter* strains, representative *Agrobacterium* spp., and rhizogenic α-*Proteobacteria* isolated from RMA crops.

three strains (CSL 2573, 2637, and 3809) formed a clade with two *Ochrobactrum* spp. One other strain (CSL 2611) formed a clade with several *Sinorhizobium* spp. The two RMA *Agrobacterium* strains were placed within a clade of other *Agrobacterium* biovar 1 strains as expected (Fig. 2).

In vitro pathogenicity test. Results from the in vitro host tests on cucumber cotyledon leaves are summarized in Table 1. Symptoms were first observed as callus formation at the midrib of the cut leaf edge, usually within 5 to 14 days postinoculation. Roots then developed from the callus tissue within a further 5 to 14 days. Roots were thick in appearance, with irregular branching (Fig. 3). Secondary callus formation along the cut edge, and sometimes on the leaf surface at the site of forceps damage, was also observed. Roots again initiated from these calli. Root induction from noninoculated controls and disks inoculated with an *Agrobacterium* sp. (CSL 3260) not possessing an Ri plasmid was occasionally observed. However, callus tissue was not associated with such disks and roots were thin in appearance, with regular branching, similar in appearance to roots observed in noninoculated cucumber cotyledons of a previous study (25). Because a proportion of cotyledons did not survive the initial tissue culture steps, leaf disks were not considered negative unless they had survived at least 14 days postinoculation. Positives were only those disks from which roots had developed from callus tissue. Occasional disks, inoculated with rhizogenic strains, on which callus tissue was observed but from which roots were not induced were considered negative.

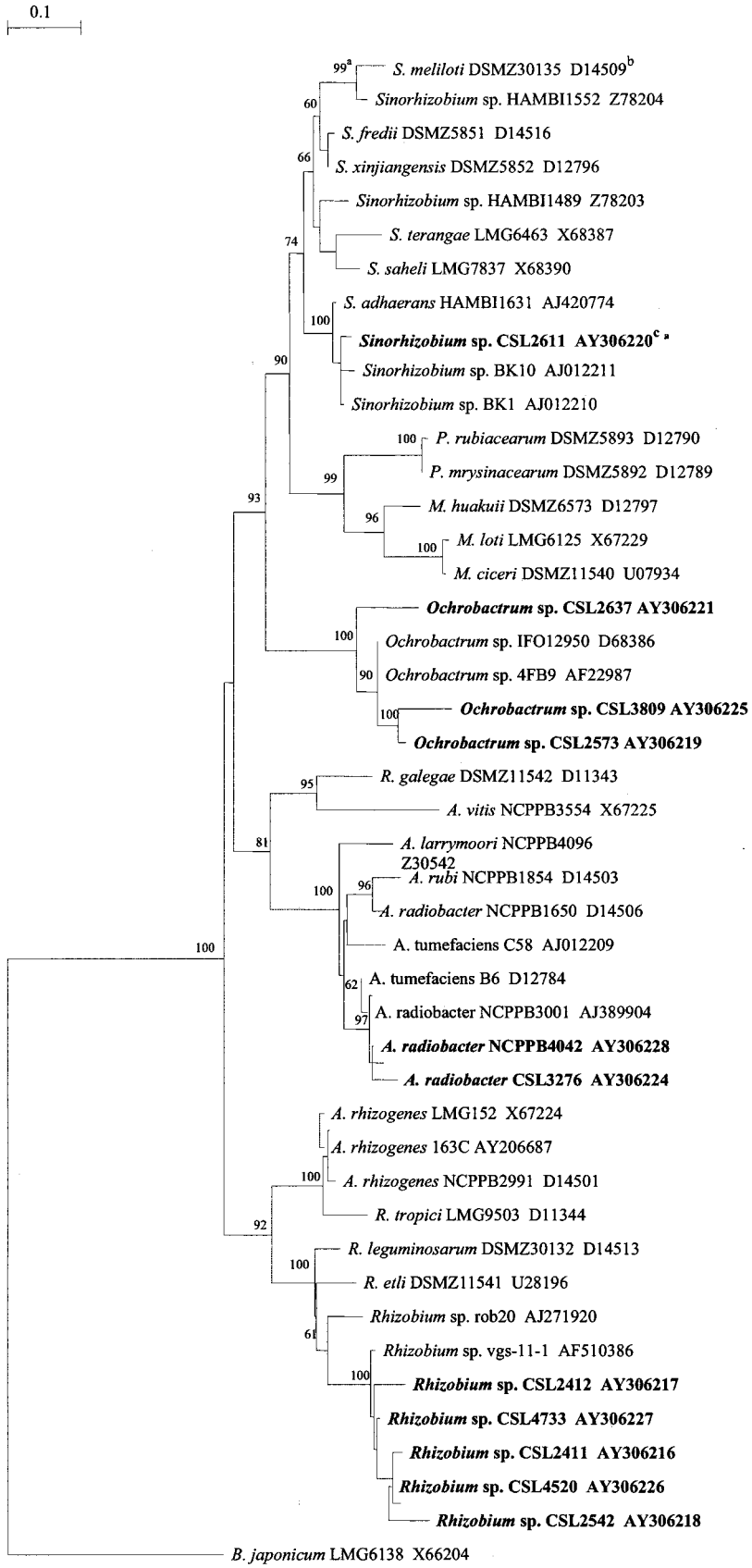
0.1
99[a]
S. meliloti DSMZ30135 D14509[b]
Sinorhizobium sp. HAMBI1552 Z78204
60
S. fredii DSMZ5851 D14516
66
S. xinjiangensis DSMZ5852 D12796
Sinorhizobium sp. HAMBI1489 Z78203
74
S. terangae LMG6463 X68387
S. saheli LMG7837 X68390
S. adhaerans HAMBI1631 AJ420774
100
Sinorhizobium sp. CSL2611 AY306220[c a]
90
Sinorhizobium sp. BK10 AJ012211
Sinorhizobium sp. BK1 AJ012210
100
P. rubiacearum DSMZ5893 D12790
99
P. mrysinacearum DSMZ5892 D12789
93
M. huakuii DSMZ6573 D12797
96
100
M. loti LMG6125 X67229
M. ciceri DSMZ11540 U07934
Ochrobactrum sp. CSL2637 AY306221
100
Ochrobactrum sp. IFO12950 D68386
90
Ochrobactrum sp. 4FB9 AF22987
100
Ochrobactrum sp. CSL3809 AY306225
Ochrobactrum sp. CSL2573 AY306219
95
R. galegae DSMZ11542 D11343
A. vitis NCPPB3554 X67225
81
A. larrymoori NCPPB4096 Z30542
96
A. rubi NCPPB1854 D14503
100
A. radiobacter NCPPB1650 D14506
100
A. tumefaciens C58 AJ012209
62
A. tumefaciens B6 D12784
97
A. radiobacter NCPPB3001 AJ389904
A. radiobacter NCPPB4042 AY306228
A. radiobacter CSL3276 AY306224
A. rhizogenes LMG152 X67224
A. rhizogenes 163C AY206687
100
A. rhizogenes NCPPB2991 D14501
R. tropici LMG9503 D11344
92
R. leguminosarum DSMZ30132 D14513
100
R. etli DSMZ11541 U28196
Rhizobium sp. rob20 AJ271920
61
Rhizobium sp. vgs-11-1 AF510386
100
Rhizobium sp. CSL2412 AY306217
Rhizobium sp. CSL4733 AY306227
Rhizobium sp. CSL2411 AY306216
Rhizobium sp. CSL4520 AY306226
Rhizobium sp. CSL2542 AY306218
100
B. japonicum LMG6138 X66204

FIG. 3. Rooting from cucumber cotyledon inoculated with rhizogenic *A. radiobacter* strain (NCPPB 4042) 12 days postinoculation.

Transformation efficiencies varied between species, with rhizogenic *Agrobacterium* strains having an average rate of nearly 64%, compared with rates of strains from the *Rhizobium* and *Ochrobactrum* clades which averaged nearly 44% and 46%, respectively. The single rhizogenic *Sinorhizobium* sp. had a transformation efficiency of 75%, although individual rhizogenic *Agrobacterium*, *Rhizobium*, and *Ochrobactrum* strains also had similarly high rates.

Analysis of root culture tissue by PCR. A DNA extract, prepared from root tissue induced from a cotyledon inoculated with the rhizogenic *A. radiobacter* strain NCPPB 4042, tested positive in each of the *fliG*, *rol*, and COX PCR assays, indicating that both plant and bacterial DNA was present in the DNA extract and implying that the cefotaxime in the MS medium suppressed rather than eliminated *A. radiobacter*. A DNA extract was prepared from a pure culture of NCPPB 4042. From this extract a 10-fold dilution series was prepared and used as template in individual *fliG*, *rol*, and COX PCRs. Mean (two replicates) threshold cycle (C_t) values, a measure of the quantity of DNA present within each sample, from this dilution series were compared with those obtained from the root DNA extract (Table 2). There is a linear relationship between C_t values for *rol* and *fliG* in the dilution series, whereas for the root tissue the *rol* threshold is reached 4.17 cycles earlier than would be predicted by this relationship. From this fact it can be estimated that the abundance of *rol* relative to *fliG* is elevated 16.3-fold in the root tissue in comparison to that of the bacterial culture, under the assumption that bacterial DNA was extracted equally from both bacterial and root cultures. It is probable that much of this increase is attributable to transformation of plant tissue with T-DNA (which carries *rol* but not *fliG*). However, it is also possible that the copy number of pRi might be higher in the bacteria that have colonized the leaf disk. Changes in pTi copy number of up to eightfold have been reported for *Agrobacterium* sp. in response to an autoinducer (20). The calculations above necessitate *fliG* C_t data. As none of the nine α-*Proteobacteria* strains are detected by the *fliG* assay (Table 1), it is not possible to extend this assay to root material induced by these strains, as it could not be proven that any increase in *rol* signal is not solely caused by an abundance of residual bacteria. As the roots induced by these strains showed morphology typical of those induced by rhizogenic *A. radiobacter* and were also induced from callus tissue, it was assumed that these roots were also transformed.

TABLE 2. Quantitative PCR demonstrating transformation of plant tissue as well as persistence of bacteria[a]

DNA extract (dilution)	Mean C_t value from PCR[b] of:		
	COX	*fliG*	*rol*
Root (undiluted)	24.64	21.32	18.50
NCPPB 4042 (undiluted)	—[c]	16.06	17.19
NCPPB 4042 (10^{-1})	—	19.30	20.72
NCPPB 4042 (10^{-2})	—	22.89	24.20
NCPPB 4042 (10^{-3})	—	25.97	27.52

[a] C_t values are from three separate TaqMan assays performed on a root DNA extract obtained from a cucumber cotyledon inoculated with NCPPB 4042 and a dilution series obtained from a pure culture of NCPPB 4042.
[b] For information on procedures for COX PCR, see Weller et al. (34); for *fliG* PCR, see Weller et al. (36); for *rol* PCR, see Weller and Stead (35).
[c] —, no fluorescence above threshold was detected.

DISCUSSION

To the best of our knowledge, this is the first report of the isolation direct from environmental samples of non-*Agrobacterium* strains which harbor an Ri plasmid. RMA crops show extensive root proliferation (32, 33). The presence of large populations of pRi-harboring *A. radiobacter* strains and quantities of cucumopine will create more favorable conditions in such a rhizosphere for both conjugal transfer and maintenance of pRi in recipient bacterial strains than those found for the spread of pTi in crown gall-affected plants, where volumes of transformed tissues and populations of pTi-harboring *Agrobacterium* strains are generally lower.

Initial fatty acid and PCR analysis identified nine pRi-harboring strains which had profiles that differed from those of a panel of RMA *A. radiobacter* strains and five *Agrobacterium* type strains. These strains appeared to be related to the genus *Agrobacterium*, and thus it was unclear whether these strains

FIG. 2. Neighbor-joining phylogeny for partial 16S rRNA sequences (alignment length, 1,050 bp) showing relationships between rhizogenic α-*Proteobacteria* isolated from RMA cucumber and tomato crops as well as a panel of representative strains. Footnotes: a, numbers on the dendrogram represent the percentages of grouping confidence calculated by bootstrap analysis (100 bootstrap replicates); b, GenBank accession numbers; c, strains in bold are the nine rhizogenic α-*Proteobacteria* strains plus two rhizogenic *A. radiobacter* strains isolated from RMA cucumber crops.

belonged to a previously undescribed *Agrobacterium* species or were in fact genuinely not *Agrobacterium* strains. A phylogenetic tree constructed from partial 16S rRNA sequence data showed that the rhizogenic strains were placed in three places within the α-*Proteobacteria* group. Five closely related strains showed closest homology with two *Rhizobium* strains (rob20 and vgs-11). Three other strains formed a clade with two *Ochrobactrum* strains (4FB9 and IFO 12950), and an individual strain was placed within the *Sinorhizobium* cluster. The tree, based on aligned sequences of around 1,100 bp in length, bears close comparison with similar trees constructed by using entire gene (1,400 to 1,500 bp) 16S rRNA sequence data (8, 27).

Although the sole use of the 16S rRNA gene for phylogenetic placement of α-*Proteobacteria* has recently been questioned (30), the sequence of some 70% of this gene, together with the results obtained from the *fliG* PCR and fatty acid profiling, allows these nine strains to be confidently placed outside the genus *Agrobacterium* and into the genera identified. However, further work would be needed in order to assign each of these strains to individual species.

Two PCR assays were employed to demonstrate the presence of pRi in these strains, one targeted to the conserved *virD2* gene (11) found in all pTi and pRi and one targeted to the T-DNA of pRi commonly associated with RMA crops (35). We have assumed that positive results in both of these assays and the subsequent results in the in vitro pathogenicity test indicate the presence of pRi in the nine α-*Proteobacteria* strains, although the presence of pRi was not demonstrated by other techniques, such as electrophoretic isolation of plasmids (14).

Teyssier-Cuvelle et al. (27) report the use of a conjugal transfer system, consisting of a counter-selectable polyauxotrophic derivative of the *Agrobacterium* sp. C58 strain and a highly transferable pTi (pSTiEGK), which demonstrated the transfer of pTi to recipient *Sinorhizobium* spp. in soil microcosm experiments. One of the strains isolated in our study (CSL 2611) had very close 16S rRNA sequence similarity to these recipient strains (BK1 and BK10). This transfer system has subsequently been used to demonstrate that pTi can be transferred to other members within the *Rhizobiaceae* group, including *Ochrobactrum* strains, but the more taxonomically remote genus *Bradyrhizobium* members could not accept the plasmid (X. Nesme, personal communication). These results support the observations of our study, i.e., pRi was not detected in strains outside α-*Proteobacteria*.

The complete nucleotide sequence of the mikimopine-type Ri plasmid pRi1724 has been determined previously (16) and has been shown to have close similarity to the cucumopine-type plasmid pRi2659, a known RMA pRi (32). The conjugal transfer (*tra*) system of pRi1724 shows closer similarity to the *tra* system of the sequenced *Sinorhizobium* Sym plasmid pNGR234 (6) and to some plasmids in *R. leguminosarum* (29) than to the *tra* systems in several Ti plasmids (16). The plasmid replication genes of *Agrobacterium* plasmids are also related to those of rhizobia (21). A *Rhizobium* symbiotic plasmid (pSymG100) has been shown to belong to the same incompatibility class as the *Agrobacterium* Ri plasmid (pRi1855) (18), indicating that a self-transmissible RMA pRi could disseminate to other α-*Proteobacteria*. Large quantities of cucumopine in the rhizosphere are likely to favor conjugation of pRi. Conjugation of pTi has been shown to be induced by the presence of other opines (7).

The small sample size of isolated rhizogenic α-*Proteobacteria* means it is not possible to determine the full taxonomic range of pRi transfer in RMA rhizospheres. The nine strains were, in effect, isolated by accident during a process designed to isolate *Agrobacterium* strains. As many α-*Proteobacteria* are uncultivable on the media used in this study, many potential transconjugants may not have been isolated. Indeed, only cultivable strains were analyzed in this report, and as more than 99% of bacteria present in environmental samples are generally considered to be uncultivable (1), we cannot be certain that pRi cannot disseminate outside α-*Proteobacteria*.

Rhizogenic *A. radiobacter* strains have nearly always been associated with RMA crops (32). The extent of symptom expression on whole plants induced by other pRi-harboring α-*Proteobacteria* in the absence of *A. radiobacter* strains has not been determined in this study. The cucumopine opine exuded from transformed tissues will likely create a bias favoring cucumopine-catabolizing bacteria (10, 19), and genes for cucumopine catabolism will be present on cucumopine-type pRi. Thus, the presence of cucumopine in the root mat rhizosphere would confer an advantage on pRi-harboring α-*Proteobacteria* even if they were unable to transform root tissues.

All isolated pRi-harboring α-*Proteobacteria* induced root mat symptoms following in vitro inoculation of cucumber cotyledons. The pathogenicity of such strains, especially in a natural environment, will not be determined solely by the acquisition of pRi. Chromosomal virulence genes, encoding functions such as bacterial attachment to the plant cell or exopolysaccharide production, are a necessary component of the *Agrobacterium* transformation mechanism (9). Similar genes, necessary for the establishment of the symbiotic relationship between nodulating α-*Proteobacteria* spp. and root tissues, have been detected within the *Sinorhizobium meliloti* chromosome (4, 15), implying that suitable chromosomal elements exist within α-*Proteobacteria* to ensure pRi-mediated transformation is possible by transconjugant strains. Experimental transfer of a pTi to *R. leguminosarum* biovar *trifolii* resulted in this strain becoming pathogenic (13), although transfer of another pTi to *S. meliloti* did not result in a pathogenic strain despite *vir* gene induction and T-DNA formation (31). This failure was possibly due to poor attachment of the bacteria to the plant cells or failure of T-DNA transfer from the bacterium and indicates that transfer of pRi may not always result in pathogenic α-*Proteobacteria* strains in root mat rhizospheres.

Although the in vitro test is limited in that leaf, rather than root, tissue is inoculated, average transformation rates were higher for rhizogenic *A. radiobacter* strains than for rhizogenic *Rhizobium* and *Ochrobactrum* strains. However, individual strains from both of these genera, plus the rhizogenic *Sinorhizobium* strain, showed higher transformation rates comparable to those seen with individual *A. radiobacter* strains. This implies that although a randomly selected pRi-harboring *A. radiobacter* strain is more likely to induce extensive symptoms, strains exist outside the *Agrobacterium* genus that are also likely to be effective pathogens.

This study has been undertaken as part of a larger project to select biocontrol agent(s) that can control root mat in hydro-

ponic crops. Ri plasmid transfer to other α-*Proteobacteria* from *A. radiobacter* complicates this choice. A biocontrol agent that specifically targets *A. radiobacter* may only lead to root mat being induced by non-*Agrobacterium* strains harboring pRi. Although we have only shown in vitro induction of root mat symptoms by such strains, the numbers and genetic diversity of potential pRi recipients suggest that there is a high probability that efficient non-*Agrobacterium* root mat inducers exist. Thus, biocontrol agent(s) with a broad range of action and protection may be the best avenue to success. Even if no efficient non-*Agrobacterium* pathogens exist, rhizogenic α-*Proteobacteria* will play an important epidemiological role in the disease. Avirulent *Agrobacterium* strains are ubiquitous both on hydroponic cucumber nurseries (32) and in nature (3), and thus pRi-harboring non-*Agrobacterium* species may act as reservoirs of pRi.

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